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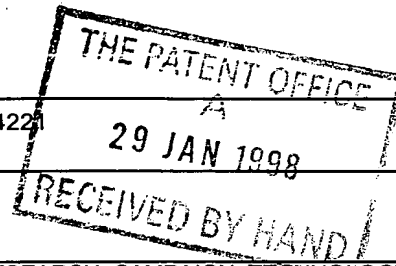
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CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED
Cambridge House
6-10 Cambridge Terrace
Regent's Park
London NW1 4JL

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

3984150002

4. Title of the invention

A GENE PROMOTER

5. Name of your agent (if you have one)

MEWBURN ELLIS

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

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A gene promoter

Field of the invention

5 The present invention relates to the cloning of a genomic promoter region of the human and mouse telomerase RNA gene. Particularly, but not exclusively, it relates to the identity of elements important for the regulation of telomerase RNA genes.

Background of the invention

10 Telomeres are found at the end of linear chromosomes and consist of short repetitive sequences essential for the maintenance of normal chromosome structure and function (Wellinger & Sen, 1997). With each cell division, 15 telomeres shorten due to the inability of DNA polymerases to replicate the ends of linear DNA molecules. However, telomere erosion is counteracted by the activity of the enzyme telomerase, a ribonuclear protein with reverse transcriptase activity, which adds telomeric repeats to the 20 chromosomal termini (Morin, 1997; Nakamura et al., 1997). The genes for the human, (hTR), and mouse, (terc), RNA components have recently been cloned, as has the human protein component, (hTRT) (Blasco et al., 1995; Feng et al., 1995; Nakamura et al., 1997; Soder et al., 1997b; 25 Soder et al., 1997c). Whilst telomerase expression is detectable in normal embryonic tissues and germline stem cells, telomerase expression is repressed in most normal postnatal somatic cells (Blasco et al., 1995; Feng et al., 1995; Prowse & Greider, 1995; Soder et al., 1997a; Wright 30 et al., 1996). The lack of telomerase expression may be the major reason for the progressive loss of telomeric sequences in somatic cells, which is considered to be one regulatory mechanism which monitors the number of times a cell divides before entering replicative senescence 35 (Campisi, 1997). However, although telomerase appears to be stringently repressed in normal somatic tissues, there is substantial evidence to suggest that telomerase is

expressed in the majority of human cancers and contributing to the immortal phenotype through the maintenance of telomere integrity (Holt et al., 1997; Kim, 1997; Shay & Bacchetti, 1997).

5 The regulation of telomerase expression is a complex issue including transcriptional activity of the telomerase RNA and protein component genes, interaction of telomerase with other telomere associated proteins and post-translational modification of the enzyme complex. However,
10 at present there are few studies which directly address the mechanisms regulating telomerase expression in normal and cancer cells (Bodnar et al., 1996; Broccoli et al., 1997; Li et al., 1997; Mandal & Kumar, 1997; Morin, 1997; Nakamura et al., 1997; Soder et al., 1997a).

15 Studies aimed at relating genome stability to human cellular senescence have recently placed considerable emphasis on telomerase expression as a central unifying mechanism underlying the immortal phenotype of many cancers (Breslow et al., 1997). The absence of telomerase activity
20 from normal somatic cells has led to the proposal that telomere shortening may be a molecular clock which contributes to the onset of cellular senescence in normal cells (Harley & Villeponteau, 1995; Holt et al., 1996). Conversely, the reactivation or expression of telomerase
25 may be a major mechanism by which cancer overcome normal cellular senescence (Kim et al., 1994; Parkinson et al., 1997). Information on telomerase activity in tumours almost exclusively derives from the *in vitro* telomere repeat amplification protocol, (TRAP), and these have shown that
30 telomerase activity may be present in greater than 80% of tumour biopsies yet absent or reduced in normal somatic tissue (Breslow et al., 1997; Kim et al., 1994; Raymond et al., 1996; Shay & Wright, 1996). However, TRAP assay alone
35 will not reveal the true complexities of telomerase regulation, and it is generally recognised that a number of molecular approaches will be required to understand telomere length regulation and telomerase activity (Breslow

et al., 1997; Holt et al., 1996; Lundblad & Wright, 1996; Parkinson et al., 1997; Raymond et al., 1996; Soder et al., 1997b). Recently, the present inventors and others have introduced a number of more direct *in situ* approaches to study the telomerase RNA gene, (hTR) and its expression in tumours (Soder et al., 1997; Yashima et al., 1997b).

US patent 5583016 (Geron Corp.) Discloses a 2.4kb sequence of the Telomerase RNA gene. However, there is no disclosure of the promoter elements or provision of functional evidence to show the promoter is active.

Summary of the invention

The levels of telomerase RNA gene expression vary during normal development and between normal and cancerous cells and tissues (Avilion et al., 1996; Bestilny et al., 1996; Blasco et al., 1995; Blasco et al., 1996; Bodnar et al., 1996; Broccoli et al., 1996; Feng et al., 1995; Kuniyasu et al., 1997; Soder et al., 1997a). The present inventors have appreciated that knowledge of telomerase RNA gene expression should aid understanding of the signal transduction pathways linking telomere attrition to proliferation, cellular senescence, differentiation and oncogenesis. As a first step towards this goal, they have cloned the promoter regions of the human, (hTR), and mouse, (*terc*), telomerase RNA genes in order to identify the regulatory elements controlling telomerase RNA gene transcription. Further, the present inventors have investigated the possible levels of telomerase regulation *in vivo*.

These studies have a number of implications for the development of new transcription based therapies for cancer (Cai et al., 1996; Connors, 1995; Miller & Whelan, 1997; Peterson & Baichwal, 1993). Directly down-regulating expression of the telomerase RNA gene through manipulation of transcription factors should be an effective anticancer therapy and the cloning of the hTR gene promoter will allow the analysis of therapeutic molecules which modulate hTR

promoter activity (Cai et al., 1996; Peterson & Baichwal, 1993; Sharma et al., 1997). Indeed, by using a human cell line which has telomerase activity, (HeLa) and one which expresses the hTR gene but is telomerase negative, (GM847), (Bryan et al., 1997), the present inventors provide a system in which the specificity of transcriptional manipulation of hTR may be examined. In comparison to HeLa, the growth of GM847 does not appear to be dependant on telomerase expression, thus transcriptional targeting of hTR in GM847 should have no cellular effects, whereas HeLa should be sensitive to the predicted anti-proliferative effects of the transcriptional targeting. In addition, the present inventors show tumour-specific patterns of hTR gene expression with clear differentials in expression between cancerous and adjacent normal tissue, (Soder et al., 1997a (not yet published); Soder et al., 1997b).

Broadly, the present invention provides materials and methods relating to the telomerase RNA (TR) gene promoter and its effects in tumour development.

According to a first aspect there is provided a nucleic acid molecule comprising the TR gene promoter.

In a second aspect there is provided a nucleic acid molecule comprising a human TR gene promoter, preferably the promoter comprising a sequence of nucleotides shown in Fig. 4a. The promoter may comprise one or more fragments of the sequence shown in Fig. 4a, sufficient to promote gene expression. In particular, it may comprise or consist essentially of a sequence of nucleotides extending at least 200 base pairs (bp), or 250 bp, 272 bp, or 300 bp, preferably at least 320 bp, more preferably at least 340 bp, even more preferably at least 400 bp upstream of the transcription start site. More preferably, the promoter may comprise a sequence of nucleotides of at least 230 bp in length between positions -272 bp and -42 bp (Fig. 4a and Fig. 5a).

In a further preferred form the promoter comprises or consists essentially of the construct designated hProm505

being a sequence of nucleotides of approximately 505 bp in length from position -463 as shown in Fig. 4 or the construct designated hProm867 being 867 bp in length from position -798 bp (Fig. 4a and Fig. 5a).

5 In a third aspect of the present invention there is provided a nucleic acid molecule comprising mouse Telomerase RNA (*terc*) gene promoter, preferably comprising or consisting essentially of the sequence as shown in Fig. 4b. In a preferred form, the present invention provides a
10 nucleic acid molecule comprising a *terc* promoter, said promoter comprising or consisting essentially of a nucleotide sequence extending at least 94 bp, preferably at least 100 bp, more preferably at least 120 bp, even more preferably at least 150 bp upstream of the transcription
15 start site. Preferably the promoter comprises or consists essentially of a nucleotide sequence of approximately 73 bp in length between -94 bp and -22 bp as shown in Fig. 4b and Fig. 5b.

In further preferred form, the present invention
20 provides a nucleic acid molecule comprising or consisting essentially of the construct designated mProm208 or the construct designated mProm628 as shown in Fig. 4 or Fig. 5b.

An even smaller portion of the nucleotide sequences
25 mentioned above may be used as long as the promoter activity is retained. Such nucleotide sequences may be fragments being 200 nucleotides or fewer in length (e.g. 150, 100, 50, 40, 35, 30, 25, or 20). Restriction enzymes or nucleases may be used to digest the nucleic acid,
30 followed by the appropriate assay (for example as illustrated herein using luciferase constructs) to determine the minimal sequence required. A preferred embodiment of the present invention provides a nucleic acid molecule with the minimal nucleotide sequence shown in Fig. 4a or Fig. 4b required for promoter activity. The minimal
35 promoter is situated between -272 bp and -42 bp as shown in Fig. 4a and between -94 bp and -22 bp as shown in Fig. 4b.

The promoter may comprise one or more sequence motifs or elements conferring developmental and/or tissue-specific regulatory control or expression. Other regulatory sequences may be included, for instance as identified by mutation or digest assay in an appropriate expression system or by sequence comparison with available information, e.g. using a computer to search on-line databases.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and orientated for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

The present invention extends to a promoter which is an allele, mutant, variant or derivative, by way of nucleotide addition, substitution or deletion of a promoter sequence as provided herein. Systematic or random mutagenesis of nucleic acid to make an alteration to the nucleotide sequence may be performed using any technique known to those skilled in the art. One or more alterations to a promoter sequence according to the present invention may increase or decrease promoter activity, or increase or decrease the magnitude of the effect of a substance able to modulate the promoter activity.

"Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with

the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction. Use of a reporter gene as discussed further below facilitates determination of promoter activity by reference to protein production.

In various embodiments of the present invention a promoter which has a sequence that is a fragment, mutant, allele, derivative or variant, by way of addition, insertion, deletion or substitution of one or more nucleotides, of the sequence of the hTR promoter shown in Fig. 4a or the *terc* promoter shown in Fig. 4b, has at least about 60% homology with one or both of the shown sequences, preferably at least about 70% homology, more preferably at least about 80% homology, more preferably at least about 90% homology, more preferably at least about 95% homology. Such homology may be found over a sequence of at least 10 nucleotides, preferably of at least 20 nucleotides, more preferably of at least 30 nucleotides and even more preferably of at least 40 nucleotides. Such fragments themselves individually represent aspects of the present invention.

The sequence in accordance with an embodiment of the invention may hybridise with one or both of the shown sequences, or the complementary sequences (since DNA is generally double-stranded). The sequence may have the ability to promote transcription (i.e. have "promoter activity") in normal embryonic tissues and germline stem cells.

On the basis of the nucleotide sequences given herein (Fig. 4a and Fig. 4b), oligonucleotide probes or primers may be designed. Generally specific primers are upwards of 14 nucleotides in length but not more than 18 to 20. Those skilled in the art are well versed in the design of primers for use processes such as PCR. Primer sequences which themselves individually form part of the present invention are given in Fig. 6.

Further provided by the present invention is a nucleic

acid construct comprising a TR promoter region or a fragment, mutant, allele, derivative or variant thereof able to promote transcription, operably linked to a heterologous gene, e.g. a coding sequence. By
5 "heterologous" is meant a gene other than TR gene. Modified forms of TR are generally excluded. Generally, the gene may be transcribed into mRNA which may be translated into a peptide or polypeptide product which may be detected and preferably quantitated following
10 expression. A gene whose encoded product may be assayed following expression is termed a "reporter gene", i.e. a gene which "reports" on promoter activity.

The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal,
15 preferably a visually detectable signal, such as a coloured product. Many examples are known, including β -galactosidase and luciferase. β -galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a spectrophotometer to
20 measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-
25 radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly
30 using any standard technique.

Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine gene activity. Any suitable reporter/assay may be used and it should be appreciated
35 that no particular choice is essential to or a limitation of the present invention.

Expression of a reporter gene from the promoter may be

in an *in vitro* expression system or may be intracellular (*in vivo*). Expression generally requires the presence, in addition to the promoter which initiates transcription, a translational initiation region and transcriptional and translational termination regions. One or more introns may be present in the gene, along with mRNA processing signals (e.g. splice sites).

The present invention also provides a nucleic acid vector comprising a promoter as disclosed herein. Such a vector may comprise a suitably positioned restriction site or other means for insertion into the vector of a sequence heterologous to the promoter to be operably linked thereto.

Suitable vectors can be chosen or constructed containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Procedures for introducing DNA into cells depend on the host used, but are well known.

Thus, a further aspect of the present invention provides a host cell containing a nucleic acid construct comprising a promoter element, as disclosed herein, operably linked to a heterologous gene. A still further aspect provides a method comprising introducing such a construct into a host cell. The introduction may employ any available technique, including, for eukaryotic cells, calcium phosphate transfection, DEAE-Dextran transfection, electroporation, liposome-mediated transfection and transduction using retrovirus.

The introduction may be followed by causing or allowing expression of the heterologous gene under the control of the promoter, e.g. by culturing host cells under conditions for expression of the gene.

In one embodiment, the construct comprising promoter and gene is integrated into the genome (e.g. chromosome) of

the host cell. Integration may be promoted by inclusion in the construct of sequences which promote recombination with the genome, in accordance with standard techniques.

5 Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992, the disclosure of which is incorporated herein by reference.

10 Nucleic acid molecules, constructs and vectors according to the present invention may be provided isolated and/or purified (i.e. from their natural environment), in substantially pure or homogeneous form, free or substantially free of a TR coding sequence, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the promoter sequence. Nucleic acid according to the present invention may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities.

15 Nucleic acid constructs comprising a promoter (as disclosed herein) and a heterologous gene (reporter) may be employed in screening for a substance able to modulate activity of the promoter. For therapeutic purposes, e.g. for treatment of cancers, a substance able to regulate the promoter may be sought. A method of screening for ability of a substance to modulate activity of a TR promoter may comprise contacting an expression system, such as a host cell, containing a nucleic acid construct as herein disclosed with a test or candidate substance and determining expression of the heterologous gene.

20 The level of expression in the presence of the test substance may be compared with the level of expression in the absence of the test substance. A difference in expression in the presence of the test substance indicates ability of the substance to modulate gene expression. A

decrease in expression of the heterologous gene compared with expression of another gene not linked to a promoter as disclosed herein indicates specificity of the substance for disrupting the TR promoter.

5 A promoter construct may be transfected into a cell line using any technique previously described to produce a stable cell line containing the reporter construct integrated into the genome. The cells may be grown and incubated with test compounds for varying times. The cells
10 may be grown in 96 well plates to facilitate the analysis of large numbers of compounds. The cells may then be washed and the reporter gene expression analysed. For some reporters, such as luciferase the cells will be lysed then analysed.

15 Constructs comprising one or more developmental and/or time-specific regulatory motifs (as discussed) may be used to screen for a substance able to modulate the corresponding aspect of the promoter activity, e.g. cancer related expression.

20 Following identification of a substance which modulates or affects TR gene promoter activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a
25 medicament, pharmaceutical composition or drug. These may be administered to individuals.

 Thus, the present invention extends in various aspects not only to a substance identified using a nucleic acid molecule as a modulator of TR gene promoter activity, in
30 accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for decreasing TR gene expression for
35 instance in treatment of cancers, use of such a substance in manufacture of a composition for administration, e.g. for decreasing TR gene expression for instance in treatment

of cancers, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Administration will preferably be in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, eg decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection,

or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Also included within the scope of the present invention are substances that disrupt the ability of the TR gene promoter as herein described to regulate expression of the TR gene. These substances include any member that is capable of directly down-regulating telomerase RNA gene expression or which specifically blocks transcriptional activation of the TR gene promoters through interaction of the 5' regulatory sequences, for example, antisense oligonucleotides, transcription factors, synthetic oligonucleotides and peptide nucleic acids (hybrid molecules between peptides and nucleic acids) or factors that disrupt signal transduction pathways.

The present inventors have devised reporter constructs, which form part of the invention, which can be introduced into cells or cell free systems to monitor promoter activity. This allows regulation of transcription and the development of small molecules specifically designed to disrupt the hTR transcriptional machinery. The reporter cell lines so produced also form part of the invention and are ideally suited to test any commercial telomerase inhibitors which may be evaluated, in combination with standard telomerase assays for specificity and mode of action. Therefore, the present invention provides methods and kits for testing telomerase inhibitors and molecules specifically designed to disrupt hTR transcriptional machinery. Further, the present invention extends to the use of these reporter constructs to monitor promoter activity and regulate transcription.

The identification of the transcription factors regulating telomerase RNA gene expression and the elucidation of the specific molecules of the signal transduction pathways regulating expression will provide a wealth of targets for anticancer agents. Inhibitors may be developed by rational design based on the molecular understanding of telomerase RNA gene expression or the screening of pharmaceutical compounds in screens based on promoter/reporter activity.

Therefore, the present invention provides a method of identifying transcription factors capable of regulating TR gene expression comprising the steps of screening for compounds which disrupt TR gene promoter activity as determined by reporter activity. Telomerase inhibitors identified by such methods as described above are also within the scope of the present invention.

The work performed by the present inventors shows examples of clear differentials in hTR expression between cancerous and adjacent normal tissue which support the possibility of effective telomerase-based therapy (Soder et al 1997b; and Table 1 below). Indeed, the presence of high levels of hTR expression in specific cancers shows that the hTR/*terc* promoter may be used for genetic therapies designed to target therapeutic genes to tumours, via tumour specific gene expression.

Therefore, the present invention provides the use of nucleic acid molecules comprising promoter, promoter constructs or fragments thereof as described above for gene therapy. This includes the preparation of a medicament for use in gene therapy.

Genetically directed prodrug therapy, (GDEPT), comprises two parts: a tumour specific promoter and the enzyme prodrug system. The properties of an optimal tumour selective transcriptional activation system can be summarized as, ideally tumour specific, only expressed in nonessential tissues, no cross-specificity with unusual but essential cell types, regulatory elements from gene cloned

and sequenced, specific transcription factor binding sites identified, enhancer and inhibitory factors understood. The telomerase RNA gene promoters according to the present invention have these characteristics.

5 The present invention therefore provides the use of nucleic acid molecules comprising promoters as described herein to drive expression of enzyme-prodrug activation systems such as viral thymidine kinase and Gancyclovir, although many other systems known to those skilled in the art may also be used. Targeted gene expression via the telomerase RNA gene promoter may also be used in gene replacement strategies for cancer therapy.

10 Genomic sequences encompassing the promoter region of hTR and *terc* have been cloned into green fluorescent protein (GFP) vectors and luciferase gene reporter vectors to assay for promoter activity, and deletion and mutation constructs have been developed. By introducing these constructs into primary cultures of keratinocytes/fibroblasts and neoplastic cell lines, which have varying levels of telomerase activity and telomerase RNA gene expression, genomic regions controlling repression or activation of hTR/*terc* expression may be identified. Human embryonic cells and murine embryonic stem cells may be used to study the developmental regulation of the expression.

25 The present invention also provides DNA binding assays for identifying transcription factors in crude cell extracts. As specific promoter regions have been identified by the present inventors, specific DNA-protein interactions may be analysed by gel mobility shift assay and DNase I footprinting which are well known techniques to those skilled in the art (Gene Transcription, eds Hames, B.D. and Higgins, S.J., IRL press, Oxford University Press, Oxford 1993). In addition, *in vitro* transcription assays for the hTR/*terc* promoters may be developed to study transcription factor interactions. Protein extracts from a) cell lines with and without telomerase RNA gene expression; b) early

and late passage primary keratinocytes and fibroblasts; and
c) tumour biopsies from patients, may be assayed for the
involvement of previously characterised transcription
factors by the use of purified transcription factors,
5 oligonucleotide competition studies and antibodies to
previously characterised factors. Should it be required,
appropriate methods for the identification of the proteins
responsible for the DNA-binding activity, such as UV
crosslinking and protein purification and gene cloning may
10 be performed. Proteins identified in this way will be
considered as targets for inhibition and also form part of
the invention.

The invention further provides a method for
transcriptional analysis using transgenic mice. Transgenic
15 mice with the human and mouse promoter/reporter constructs
may be generated. Such transgenic mammals form part of the
present invention. Also, the invention provides a method of
creating transgenic mammals comprising the steps of
injecting constructs into eggs of the mammal and re-
20 implanting the eggs into the female mammal. Ideally the
mammal will be a mouse although those skilled in the art
will readily contemplate the use of other mammals such as
rats or rabbits. Mice carrying constructs in the germline
may be used to establish transgenic lines. The invention
25 also includes the use of human or mouse promoter/reporter
constructs for the generation of transgenic mammals. The
use of transgenic mice for the analysis of transcription
factors has a number of advantages over tissue culture cell
line models, for example, cell types not easily maintained
30 in culture may be studied; gene expression during animal
development may be studied; relevant mouse models of human
disease may be developed; reporter mice may be crossed with
mouse strains with other transgenes or mouse knockouts to
evaluate the biological effects of these genes on
35 telomerase RNA gene promoter activity; and compounds or
molecules thought to modulate promoter activity can be
tested *in vivo*, thus taking into account pharmacology of

the test compounds. This may be important if modulators of promoter activity are to be used clinically.

5 In a further aspect, the present invention provides a method for identifying genes regulating telomerase RNA gene expression comprising the step of introducing reporter constructs stably into cell lines and single chromosomes into the cell lines via microcell-mediated chromosome transfer (England, N.L. et al Carcinogenesis Vol. 17(8) pp. 1567-1575, 1996). This allows for fluctuations in reporter gene activity due to genes on the single chromosome to be monitored. This will identify genes carrying regulators of promoter expression and thus the signal transduction pathway controlling telomerase RNA gene transcription. Gene products identified in this way will be considered as targets for inhibition and form part of the present invention.

10 The present invention provides methods for identifying genes regulating telomerase RNA gene expression comprising the steps of introducing reporter constructs stably into cell lines and expression libraries transfected into the reporter lines. The expression libraries may be made from cells known to repress or activate telomerase activity and telomerase RNA expression. Fluctuations in reporter activity due to genes in the expression library may then be monitored, and the genes cloned. Gene products identified in this way will be considered as targets for inhibition and form part of the present invention.

20 In a further aspect, the present invention provides a method for testing candidate genes for promoter regulating activity, comprising the steps of transfecting candidate genes into cell lines containing the promoter/reporter constructs and monitoring fluctuations in reporter activity due to said candidate genes.

25 The present invention extends to the use of the TR gene promoter for any of the methods given above. This includes the use of any promoter/reporter constructs, preferably those identified in Fig 4 and Fig. 5.

Aspects and embodiments of the present invention will now be further described by way of example only with reference to the accompanying drawings. Further aspects of the invention will be apparent to those or ordinary skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Brief description of the drawings.

Figure 1 shows the 1765bp genomic nucleotide sequence (SEQ I.D. No. 1) of human telomerase RNA gene (hTR) encompassing the gene promoter region. Transient expression of hTR-reporter gene constructs in HeLa and GM847 cells identified indicate that the elements responsible for promoter activity are contained in a 231bp region upstream of the transcriptional start site.

Figure 2 shows the 4044bp genomic nucleotide sequence (SEQ I.D. No. 2) of mouse telomerase RNA gene (*terc*) encompassing the gene promoter region. Transient expression of *terc*-reporter gene constructs in SWISS3T3 and A9 cells identified the elements responsible for promoter activity are contained in a 73bp region upstream of the transcriptional start site.

Figure 3 shows the restriction enzyme map of the genomic clones encompassing the human and mouse telomerase RNA genes. The transcribed regions of hTR and *terc* are depicted as black boxes within the central regions of the genomic sequences and the site of the template sequence within the telomerase RNA genes is indicated. The position of the CpG islands are shown as a box beneath the genomic sequence. Numbers in brackets refer to the nucleotide position within the sequence. The 3'-end of all the human promoter fragments is shown as hProm and fragments extend 5-prime to hProm867, hProm697, hProm341 and hProm111. The 3'-end of all the mouse promoter fragments is shown as mProm and fragments extend 5-prime to mProm628, mProm458, mProm418, mProm267, mProm208, mProm136. The numbers after the prefix, hProm or mProm refer to the number of

nucleotides of genomic sequence contained in the promoter fragment.

Figure 4 shows the nucleotide sequence of the human, (a), and mouse, (b), telomerase RNA gene 5'-flanking regions. Putative regulatory motifs are underlined. Arrows indicate the transcriptional start sites, (Blasco et al., 1995; Feng et al., 1995) and numbers to the left of each Figure refer to the number of bases upstream of the transcriptional start site. The template regions are in bold and underlined. Sequences contained in promoter constructs are shown by vertical lines and labelled, hProm or mProm, (see Figures 1 and 3). The regions containing elements responsible for minimum promoter activity are highlighted in bold, (see text for details). The run of CpA dinucleotide repeats in the mouse promoter is shown in bold and italic.

Figure 5 shows the detection of promoter activity in the 5'-flanking regions of human and mouse telomerase RNA genes. For each construct, the length of sequence upstream from the transcriptional start site is shown to the left and the luciferase activity to the right. a) Diagram comparing luciferase activity from human promoter constructs in GM847 and HeLa cells. Data for each construct is plotted as a percentage of the hProm505 luciferase activity as this construct consistently gave the highest activity in human cells. For each construct the mean and standard deviation for duplicate transfected wells is shown. b) Diagram comparing luciferase activity from mouse promoter constructs in SWISS3T3 and A9 cells. Data for each construct is plotted as a percentage of the mProm458 luciferase activity as this construct consistently gave the highest activity in mouse cells. For each construct the mean and standard deviation for duplicate transfected wells is shown.

Figure 6 shows details of the oligonucleotide primers used for both the human and the mouse sequences. The positions of the primers can be seen on the maps provided

in Figures 7 and 8.

Figure 7A shows a map of the 1765bp hTR construct with the position of the primers (see Fig. 6) shown. In each case the percentage homology of the primer is indicated.

5 Figure 7B shows a map of the 1765bp hTR construct with the restriction sites marked.

10 Figure 8A shows a map of the 4044bp mouse *terc* construct with the position of the primers (see Fig. 6) shown. In each case the percentage homology of the primer is indicated.

Figure 8B shows a map of the 4044bp mouse *terc* construct with the restriction sites marked.

15 Figure 9 shows the sequence of the TR gene promoter from Balb/c clones. The sequence analysis shows that the sequence is identical to that of the P1 sequence (Fig. 4b) apart from minor polymorphisms.

Detailed description

20 Preparation of tissue sections for in situ hybridisation

Formalin fixed paraffin embedded tissue blocks were obtained from Pathology Department files. Tissue sections were deparaffinised, rehydrated through graded concentrations of ethanol, (100%, 90%, 70%, 50%, 30% EtOH, 25 10 sec. each), rinsed in 0.85% sodium chloride for 5 minutes, followed by PBS for 5 minute. Sections were fixed in 4% paraformaldehyde/PBS for 20 minutes, rinsed in PBS, and treated with proteinase K (40µg/ml) in 50mM Tris-HCl pH 7.5, 5mM EDTA for 7.5 minutes at room temperature. After 30 rinsing for 5 minutes in PBS, sections were post fixed in 4% paraformaldehyde/PBS for 5 minutes, rinsed in water, and acetylated in freshly prepared 0.25% acetic anhydride/0.1M triethanolamine for 10 minutes. The slides were rinsed in 0.85% saline, followed by PBS for 5 minutes each and 35 dehydrated in gradually increasing concentrations of ethanol prior to hybridisation.

Probe preparation for in situ hybridisation

The riboprobe plasmid containing telomerase RNA sequences used for RNA in situ hybridisation is as previously described (Soder et al., 1997b). Control riboprobes were human histone H3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (Ambion, Texas). The probes were labelled with (³⁵S)-UTP using a RNA labelling kit (Amersham, UK). Transcripts were purified using a Sephadex G-50 column (Pharmacia), phenol/chloroform extracted and precipitated in ethanol. The probes were resuspended in 50 mM dithiothreitol. Northern blot analysis of normal human tissue confirmed the specificity and sensitivity of the hTR probe to detect hTR expression in normal testis, (data not shown).

Hybridisation and washing procedures

Sections were hybridised overnight at 52°C in 60% formamide, 0.3M NaCl, 10mM Tris-HCl (pH 7.5), 5mM EDTA, 10% dextran sulphate, 1xDenhardtts (0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.5mg/ml yeast tRNA, 50mM DTT (freshly added), and 50 000 cpm/ul ³⁵S-labelled cRNA probe. The tissue was washed stringently at 50°C in 5x SSC, 0.1% β-mercaptoethanol for 25 minutes, at 65°C in 50% formamide, 2x SSC, 1% β-mercaptoethanol for 25 minutes, and washed twice at 37°C in 0.5M NaCl, 10mM Tris-HCl pH 7.5, 5mM EDTA for 15 minutes before treatment with 20μg/ml RNaseA at 37°C for 30 minutes. RNase A only digests single stranded RNA. This removes single stranded, and therefore unhybridised probe, leaving the RNA:RNA duplexes intact. Thus this step helps reduce background probe signal. Following washes in 50% formamide, 2xSSC, 1% β-mercaptoethanol at 65°C for 20 minutes, and twice in 2x SSC at 50°C for 15, the slides were dehydrated and dipped in 0.1% gelatine/0.01% chromealum, and then in Hypercoat Nuclear LM-1 emulsion (Amersham) and exposed for 2 weeks in light tight boxes with desiccant at 4°C. The microautradiographs were developed in 20% Phenisol for 2.5 minutes, washed in 1%

acetic and water each for 30 seconds, fixed in 30% sodium thiosulphate for 5 minutes, rinsed in water 30 minutes, and counter-stained with haematoxylin.

5 Cloning of sequences encompassing the human and mouse telomerase RNA genes

 The present inventors have previously reported the identification of genomic clones in P1 vectors containing hTR and *terc* transcribed sequences, (Soder et al., 1997b; Soder et al., 1997c). The human P1 clone, 9913, is derived from a human foreskin fibroblast P1 library and the mouse P1 clone, 11792, is derived from a mouse C127 fibroblast P1 library. Briefly, in order to subclone the promoter regions, the P1 clones were digested with *EcoRI* and *HindIII* and ligated into pBluescript. Colonies containing telomerase RNA gene sequences were identified by hybridisation with PCR generated probes as previously described, (Soder et al., 1997b; Soder et al., 1997c). Plasmid DNA was prepared from positively hybridising colonies, and inserts sequenced on both strands by dideoxy chain termination using the ABI PRISM dye terminator cycle sequencing kit (PE Applied Biosystems, Warrington, UK) and 25ng oligonucleotide primers. Dye labelled products were resolved and detected using the Applied Biosystems DNA sequencer ABI373. Sequence was analysed using the Sequencing Analysis program v3.0. Homology searches carried out using BLAST, (Basic Local Alignment Search Tool), National Centre for Biotechnology Information, (NCBI): <http://www.ncbi.nlm.nih.gov/>. Sequence was analysed for potential transcription factor binding sites by TESS: Transcription Element Search Software on the WWW, Jonathan Schug and G. Christian Overton, Technical Report CBIL-TR-1997-1001-v0.0, of the Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, 1997, <http://agave.humgen.upenn.edu/tess/index.html>. Identification of CpG islands was carried out using GRAIL: Gene Recognition and Assembly

Internet Link, <http://compbio.ornl.gov/Grail-1.3/>. The full sequences have been submitted to GenBank.

Construction of luciferase reporter gene constructs

5 The structures of the telomerase RNA gene-luciferase constructs used in the study are shown in Figures 5 and 4. Promoter-luciferase constructs were made by inserting PCR products into pGL3-Basic, (Promega). Orientation and sequence of each insert was checked by sequencing. Details
10 of the primers are given in Figure 6.

Transfection and luciferase assays

15 All transfections were carried out in duplicate in 6-well plates, (35mm diameter). Cells were seeded at 6×10^4 cell per well and cultured overnight. Transfection was carried out using SuperFect Transfection Reagent, (Qiagen), according to the manufacturers instructions. Cells were exposed to the transfection mix for three hours and harvested for analysis after 48 hours. Equivalent amounts
20 of cellular protein as determined by Bio-Rad assay, (BioRad), were used in the luciferase assay. Luciferase assays were performed according to the manufacturers protocols, (Promega). To ensure reproducibility in the assays, particular care was taken over the following: DNA
25 used for transfection was quantified by spectrophotometry and direct visualisation by gel electrophoreses. All transfections were carried out in duplicate wells and this was found to be a good measure of the reproducibility of transfection. In each experiment, all deletion constructs
30 were analysed together with both the basic cloning vector, pGL3-Basic and the positive control vector, pGL3-Control, which contains SV40 promoter and enhancer sequences. Each extract was measured for luciferase activity at least twice. All transfections were carried out at least three
35 times. Initial transfection conditions were determined by using promoter fragments linked to a green fluorescent protein reporter gene, (Clontech), as this allowed direct

visualisation of promoter activity in live cells, (data not shown). The present inventors found it important to transfect and analyse the cells at sub-confluence and that it was important to avoid harsh transfection protocols such as electroporation, resulting in poor cell viability.

Tumour specific regulation of telomerase RNA gene expression visualized by in situ hybridization.

The patterns of hTR expression were examined in epithelial cancer of lung, ovary, breast and cervix, (Table 1). Twenty six percent of non-small cell lung cancers, (NSCLC), were hTR positive. However, the NSCLC group consists of squamous, adenocarcinoma and large-cell anaplastic variants. Interestingly however, expression was almost exclusively limited to the squamous variants, ($p=0.006$), with 41% of squamous NSCLC expressing hTR but only 8% of adenocarcinoma and large-cell anaplastic NSCLC expressing hTR. This data suggests that hTR may be differentially regulated during the oncogenesis of squamous and non-squamous NSCLC. Indeed, the low frequency of detectable hTR expression in adenocarcinoma of the lung was also observed in adenocarcinomas of ovary and breast, (Table 1). In addition, metastatic carcinoma in hilar lymph nodes of 19 of the NSCLC cases were available for a comparative study with the paired primary carcinomas. All 6 cases which expressed hTR in the primary carcinoma retained expression in the metastasis and all 13 primary carcinomas which lacked detectable hTR expression remained negative in the metastasis, (Table 1). Thus, expression levels appear stable between primary and metastatic carcinomas and expression of hTR is not associated with metastasis of pulmonary carcinomas.

Cancer of the uterine cervix is a heterogeneous group of lesions, which like NSCLC can be subdivided into squamous and adenocarcinoma (Benda, 1994). The present inventors studied 87 cervical lesions for hTR expression, (Table 1). hTR expression was detected in 44% of the

cervical carcinomas, however in contrast to NSCLC, there was no significant difference in frequency of expression between invasive squamous carcinoma, (44%), and invasive adenocarcinoma, (32%). The data for adenocarcinoma of the cervix also contrast those for invasive adenocarcinoma of the breast, (13%), and ovary, (17%), (Table 1), and suggest that regulation of hTR expression may be different for cervical cancer and therefore relate to the aetiology of the disease (Benda, 1994; Klingelhutz et al., 1996). Interestingly, hTR expression was readily detected in preinvasive cervical cancer, (40%, see Table 1), and there was no significant difference in frequency between invasive and preinvasive lesions. In addition, the case of glandular intraepithelial neoplasia of the cervix has heterogeneous expression of hTR (data not shown), thus allowing the evolution of hTR expressing cells to be followed in their histological context.

The primitive germ cells of the male are found in the seminiferous tubules and the present inventors examined 22 sections from normal testis and uninvolved tubules from testicular cancer patients to establish the pattern of hTR expression in normal seminiferous tubules, (data not shown). Of the 22 sections, 21 showed hTR expression in the primitive germ cells located in the basal layers of the seminiferous epithelium, (Table 1). The intimate relationship between germ cells and the supportive Sertoli cells, means that expression of hTR in the Sertoli cells cannot be excluded. Mature germ cells (spermatids and spermatazoa) when present, did not express hTR. Thus, the *in situ* assay can detect normal levels of hTR expression in primitive germ line stem cells and the distribution of hTR expression in the testis is consistent with its proposed role in the maintenance of telomere length in the germ line. A series of 22 testicular germ cell tumours were also analysed for hTR expression. As shown in Table 1, 73% of testicular germ cell tumours were positive for hTR expression, and there was no significant difference between

teratomas and seminomas of the testis. Interestingly, within hTR-positive teratomas, mature tissues never had detectable hTR expression whatever their differentiation. Similarly, 15 benign ovarian teratomas composed of fully mature differentiated tissues had no detectable hTR expression, (Table 1). Thus, the differentiated teratomas may recapitulate the down regulation of hTR during early embryonic or foetal development, (Wright et al., 1996). In five of the testicular seminomas there was no detectable hTR expression, (Table 1), but in each seminoma case, germ cells within adjacent normal seminiferous tubules expressed hTR, suggesting either that hTR expression has been repressed during oncogenesis, or that seminomas without detectable levels of hTR arise from germ cells with low or no hTR expression.

Table 1. Frequency of hTR expression.

Tumour	Tissue histology	Frequency	%
Testicular Germ Cell Tumours	<i>normal testicular germ cells</i>	21/22	95
	^a teratoma	7/8	
	seminoma	8/13	
	intratubular	1/1	
	Total	16/22	73
Ovarian Germ Cell Tumours	^b <i>benign ovarian teratomas</i>	0/15	0
Non-small Cell Lung Cancer	squamous	13/32	41
	adenocarcinoma (18)/large cell (7)	2/25	8
	^c Total	15/57	26
Epithelial Ovarian Cancer	^d <i>adenocarcinoma</i>	6/34	17

5	Breast Cancer	^e invasive adenocarcinoma	7/54	13
		ductal carcinoma <i>in situ</i> , (DCIS)	0/19	0
		^f DCIS, recurrent	1/4	
		Phylloides	0/6	
		Total	8/83	10
10	Cervical Cancer	invasive squamous	15/34	44
		invasive adenocarcinoma	7/22	32
		^g invasive adenosquamous	7/11	
		Invasive Total	29/67	43
15		squamous intraepithelial neoplasia	4/5	
		glandular intraepithelial neoplasia	2/10	
		^h mixed squamous/glandular intraepithelial neoplasia	3/5	
		Preinvasive Total	8/20	40
20		ⁱ All cervix samples	38/87	44
	Metastasis:	positive in primary & metastasis	6/6	
	Non-small Cell Lung Cancer	negative in primary & metastasis	13/13	

Table 1. Frequency of hTR expression. ^aThe single teratoma without detectable hTR expression was of intermediate maturity and also expressed GAPDH, (data not shown), and therefore the lack of expression is not due to loss of RNA from the sample. ^bTo test for RNA integrity in the ovarian teratomas, a group of 6 were analysed for GAPDH expression and all were found to be positive. ^cA group of 33 NSLC were tested for histone H3 expression, 22 of which did not have detectable levels of hTR expression and all 33 were positive. An additional 4 hTR negative NSCLC samples tested for GAPDH expression were positive. ^dTwo ovarian cancers, (1 hTR negative), were tested for histone H3 expression and both were positive. Three ovarian cancers were tested for GAPDH expression and all were positive.

5 "A group of 10 invasive breast cancers, (7 hTR negative), were tested for histone H3 expression and all were positive. In addition, 11 breast cancers lacking detectable levels of hTR expression were tested and were positive for GAPDH expression. 'Four specimens of DCIS were obtained from patients who had in the previous 5 years been diagnosed and treated for the presence of preinvasive breast cancer, (DCIS). The primary biopsies for all 4 were lacking detectable levels of hTR expression, however one recurrent DCIS biopsy had detectable hTR expression. "Adenosquamous cancers of the cervix. Where samples were positive for hTR expression, all elements were positive. "Preinvasive cervical cancer. In lesions with mixed glandular and squamous elements, where samples were positive for hTR expression, all elements were positive. 'Two cervical cancers, (1 lacking detectable levels of hTR expression), were tested for histone H3 expression and both were positive. Three cervical cancers were tested for GAPDH expression and all were positive. In total, 28 samples, (25 lacking detectable levels of hTR expression), were tested and were positive for GAPDH expression and 47 samples, (31 lacking detectable levels of hTR expression), were tested and were positive for histone H3 expression. Visual inspection suggested that histone H3-positive cells were present in comparable numbers in the carcinomas lacking detectable levels of hTR expression and in the hTR positive carcinomas. The same areas of tissue were examined for both hTR and histone H3 expression, thus, the lack of detectable hTR expression is unlikely to be due simply to the presence of quiescent cells in the tumours, (Holt et al., 1996b).

25 Cloning of genomic sequences encompassing the human and mouse telomerase RNA genes.

In order to obtain sequences flanking the genes, the P1 genomic clones were digested with *EcoRI* and *HindIII*, subcloned into the plasmid vector, pBluescript, and colonies containing hTR or *terc* sequences identified by hybridisation to PCR generated probes specific for the genes. A 1.3kb genomic clone encompassing hTR was isolated as was a 4kb genomic clone encompassing *terc*, (Figure 3).

35 A BLAST search using the 1.3kb human sequence identified three high-scoring segment pairs: HSU85256, HSU86046 and MMU33831. HSU85256, (598bp of sequence), and HSU86046, (545bp of sequence), are published sequences for the transcribed region of the human telomerase RNA gene and confirmed that we had cloned genomic sequences encompassing hTR (Bryan et al., 1997; Feng et al., 1995). MMU33831 is the sequence of the transcribed region of the mouse telomerase RNA gene which has previously been shown to have homology to the human gene (Blasco et al., 1995).

A BLAST search using the 4kb mouse sequence identified both the published human gene sequences, (HSU85256, HSU86046), and the published sequence for the transcribed region of the mouse gene, MMU33831, (591bp of sequence) (Blasco et al., 1995; Bryan et al., 1997; Feng et al., 1995). In order to confirm that the genomic sequence obtained from the P1 subclone was genuine, the present inventors cloned 5'-flanking sequences using genomic DNA from Balb/c mice in PCR reactions. Sequence analysis of the Balb/c clones were identical to the P1 sequence except for minor polymorphism's, (Fig. 9). A schematic representation of the 4kb of sequence information encompassing the mouse telomerase RNA gene is shown in Figure 3.

Analysis of nucleotide sequence encompassing the human and mouse telomerase RNA genes.

To investigate the relationship between the human and mouse genomic clones, sequence comparisons were carried out. The transcribed regions of the two genes showed 67% identity in keeping with the published estimate, (Feng et al., 1995). However, no significant sequence identity could be identified in either the 5'- or 3'-regions flanking the transcribed sequences.

Both the human and mouse sequences were analysed for CpG islands by GRAIL. CpG islands were defined as regions larger than 200bp, with an average GC content greater than 50% and the ratio of observed versus expected CpGs greater than 0.6 (Gardiner-Garden & Frommer, 1987). Interestingly, both the human and mouse genes lie within CpG islands, (see Figure 3). The human gene is covered by a CpG island 733bp in length, with a GC content of 66% and a ratio of observed versus expected CpGs of 0.89. The mouse gene is covered by a CpG island of 659bp in length, with a GC content of 64% and a ratio of observed versus expected CpGs of 0.81.

The 5'-flanking regions of the human and mouse telomerase RNA genes were also analysed for potential

transcription factor recognition sites. As shown in Figure 4, a number of potential binding sites can be identified, including consensus sequences for glucocorticoid/progesterone/androgen receptor binding, AP1 and Ets family members. CCAAT box's are found in both genes close to the published transcriptional start sites (Blasco et al., 1995; Feng et al., 1995), however, there is no obvious TATA box in the mouse gene with the human gene TATA box consensus sequence being in the reverse orientation. The mouse promoter region also contains a run of CpA dinucleotide repeats which may of use in developing microsatellite genetic markers for this gene.

Transfection assays detect promoter activity in the 5'-flanking regions of the human and mouse telomerase RNA genes.

To identify whether the 5'-flanking DNA of the telomerase RNA genes exhibited promoter activity, sequences were fused to a firefly luciferase reporter gene, (pGL3-Basic). The transcriptional start sites for both the human and mouse telomerase RNA genes have been established (Blasco et al., 1995; Feng et al., 1995). Various promoter constructs containing the transcriptional start site were therefore generated, (see Figures 4, 5).

Human promoter constructs containing truncated portions of the 5'-flanking region were transiently transfected into HeLa and GM847 cells, (Figure 5a). HeLa is a telomerase positive cervical carcinoma cell line, GM847 is a SV40-immortalised skin fibroblast cell line which expresses the telomerase RNA component but is telomerase-negative, (Bryan et al., 1997). As shown on Figure 5a, promoter activity was observed in both cell lines with fragments containing 341bp or more, (from position -272, see Figure 4a, 5a). The highest luciferase activity was observed with construct hProm505 which contains a 505bp fragment, (position -463, see Figure 4). Construct, hProm111, which contains only 111bp of 5'-

flanking sequence, (position -42, see Figure 4a, 5a), produced a dramatically reduced level of luciferase activity, (Figure 5a). Thus, a minimal promoter sequence can be defined as extending 272bp upstream of the transcription start site, and that elements responsible for promoter activity must be contained in a 231bp region between -272bp and -42bp, (Figures 4a and 5a).

Mouse promoter constructs containing various truncated portions of the 5'-flanking region were transiently transfected into SWISS3T3 and A9 cells, (Figure 5b). SWISS3T3 cells are an embryo derived line and A9 cells are of areolar and adipose origin. Both cell lines are telomerase positive. As shown on Figure 5b, promoter activity was observed in both cell lines with fragments containing 208bp or more, (from position -94, see Figure 4b). Construct mProm136, which contains only 136bp of 5'-flanking sequence, (position -22, see Figure 4b), produced dramatically reduced levels of luciferase activity, (Figure 4b). Thus, a minimal promoter sequence can be defined as extending 94bp upstream of the transcription start site, and that elements responsible for promoter activity must be contained in a 73bp region between -94bp and -22bp, (Figure 4b and 5b).

Transfection of the human promoter construct hProm867 into mouse cells gave very strong promoter activity, with up to twice that of the strongest mouse construct, and transfection of the mouse promoter construct, mProm628 into human cells also showed luciferase activity at around 25% of the strongest human construct, (data not shown).

Discussion

In general, there are no significant sequence homologies between the promoter regions of the human and mouse telomerase RNA genes. Indeed, there is considerable debate as to whether telomere length is regulated in a similar fashion in humans and mouse (Blasco et al., 1997; Kipling, 1997a; Kipling, 1997b; Zakian, 1997). However,

mouse models represent a valuable resource with which to study the role of telomerase in cellular senescence and tumour progression and mouse models are likely to be required to investigate new therapies based on telomerase inhibition. In addition, the developmental regulation of telomerase will be more easily approached in mice (Bestilny et al., 1996; Blasco et al., 1995; Blasco et al., 1997; Blasco et al., 1996; Broccoli et al., 1996; Prowse & Greider, 1995). Thus, any differences between the two species may in fact aid understanding of the function for telomerase in maintaining genome stability and will be important in developing good murine models for human disease or developmental processes.

Despite the lack of sequence similarity between the human and mouse telomerase RNA gene promoter regions, they both have consensus sites for the binding of transcription factors implicated in haematopoiesis and leukaemogenesis such as GATA-1, PU.1, PEA2/PEBP2, C/EBP, and c-Ets-2 (Tenen et al., 1997). This data will therefore be used in the detection of telomerase activity in normal and malignant haematopoietic cells (Bodnar et al., 1996; Cheng et al., 1997; Holt et al., 1997; Norrback & Roos, 1997; Pan et al., 1997). The human and mouse telomerase RNA genes do share an interesting similarity, in that they both lie in CpG islands, and thus their expression may be regulated by methylation. DNA methylation is thought to be important for gene regulation during normal development and cellular senescence, and abnormal methylation patterns may be a fundamental change in tumour progression (Baylin et al., 1991; Bird, 1996; Laird & Jaenisch, 1996; Vertino et al., 1994; Wilson & Jones, 1983). Thus it has been suggested that aberrant CpG island methylation during the normal ageing process, could contribute to immortalisation by interfering with expression of "mortality" genes, of which hTR and *terc* can be included (Vertino et al., 1994; Wilson & Jones, 1983).

Turning to the functional analysis of the cloned

sequences, the minimal promoter for hTR resides within a region of 272bp upstream of the published transcriptional start site, (Feng et al., 1995), (Figures 5a, 4a). There are a number of potential transcription factor binding sites in this region including consensus sequences for AP1, Sp1, PEA2/PEBP2, PEA3 and PU.1. Interestingly, the expression in the fos/jun family of proteins, which determine AP1 activity, are suppressed during the onset of senescence and would be predicted to lead to a reduction in AP1 activity in senescent cells (Campisi, 1997; Irving et al., 1992; Riabowol et al., 1992; Seshadri & Campisi, 1990). AP1 also responds to protein kinase C, and it has recently been demonstrated that hTR expression is induced by protein kinase C during T-cell activation (Bodnar et al., 1996). Extending the promoter region to 463bp upstream of the transcriptional start site, increases the luciferase activity to its maximum level, (Figures 5a, 4a). This region contains several consensus binding sites for glucocorticoid/progesterone/androgen receptor binding, which may contribute to the maximal activity demonstrated by hProm505. A reduction in promoter activity is observed on extending the promoter fragments to include more 5'-sequence, (Figure 5a), suggesting that sequences towards the 5'-end of the clone may influence promoter activity in a negative fashion.

The minimal promoter for *terc* resides in a 94bp region upstream of the published transcriptional start site, (Blasco et al., 1995), (Figures 5b, 4b). A striking feature of this region is the presence of three AP-2 consensus sites, two of which are coupled to c-Ets-2 sites and all these elements are contained in the 73bp region required for promoter activity, (Figures 5b, 4b). Oncogenic Ras gene signalling has been shown to operate through c-Ets-2 binding sites, thus there is a testable relationship between oncogene activation during tumour progression and telomerase RNA gene transcriptional activity (Galang et al., 1994; Wasylyk et al., 1994). A reduction in promoter

activity is observed on extending the promoter fragments to include more 5'-sequence, (Figure 5b, mProm628), suggesting that sequences towards the 5'-end of the clone may influence promoter activity in a negative fashion.

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Fig 1

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Fig. 2

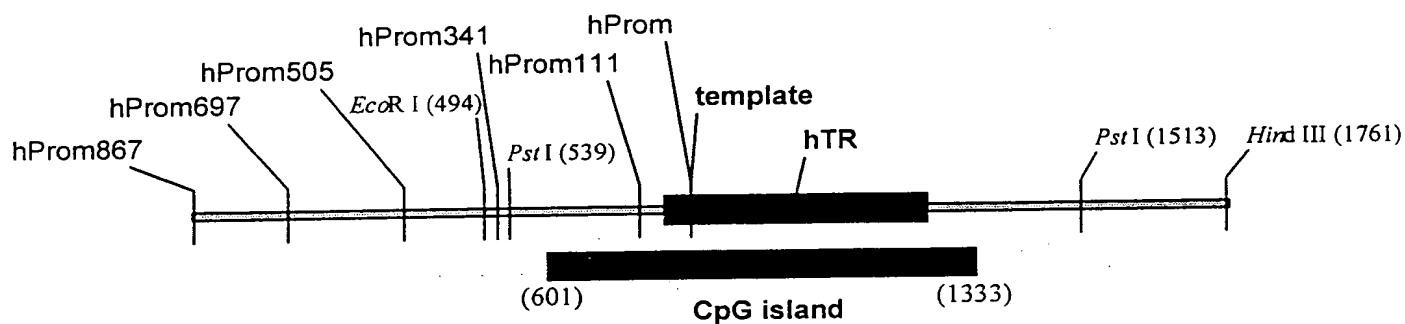
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a) Human telomerase RNA gene, hTR, genomic structure, 1765bp.



b) Mouse telomerase RNA gene, *terc*, genomic structure, 4044bp.

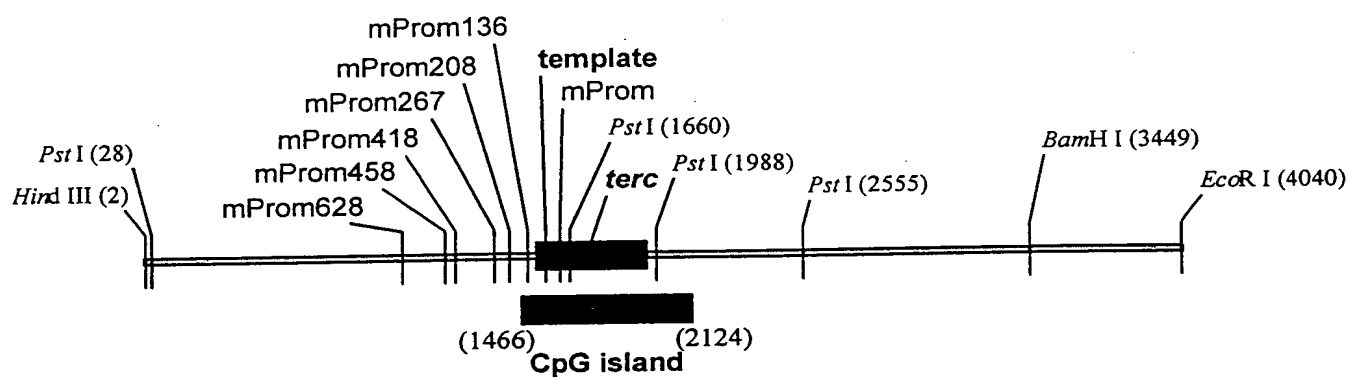


Fig. 3

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a)

Sequence of the human telomerase RNA gene promoter region

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^{AFPI/BRN2}
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^{hProm505} ^{PEA3/c-Ets-2} ^{Sp1/NF-E2} ^{CMYB}
-448 cggacgcattccctcctgagcccgagacaagattctgtctgtcagtcgtgc
^{Zeste} ^{GCN4/AP1}
-398 ctgggaatctattttcacaaagttctccaaaaatgtgatgatacaaaact
^{myogenin} ^{GR}
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^{Sp1}
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^{hProm111} ^{CCAAT Box} ^{GCN4/AP1}
-48 cggtcggcgcgcctcccttataagccgactcgcccgagcgcaacggy
^{PEA2/PEBP2} ^{GAGA} ^{TBP/TFIID}
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taactgagaagcgctal ^{hProm} template

b)

Sequence of the mouse telomerase RNA gene promoter region

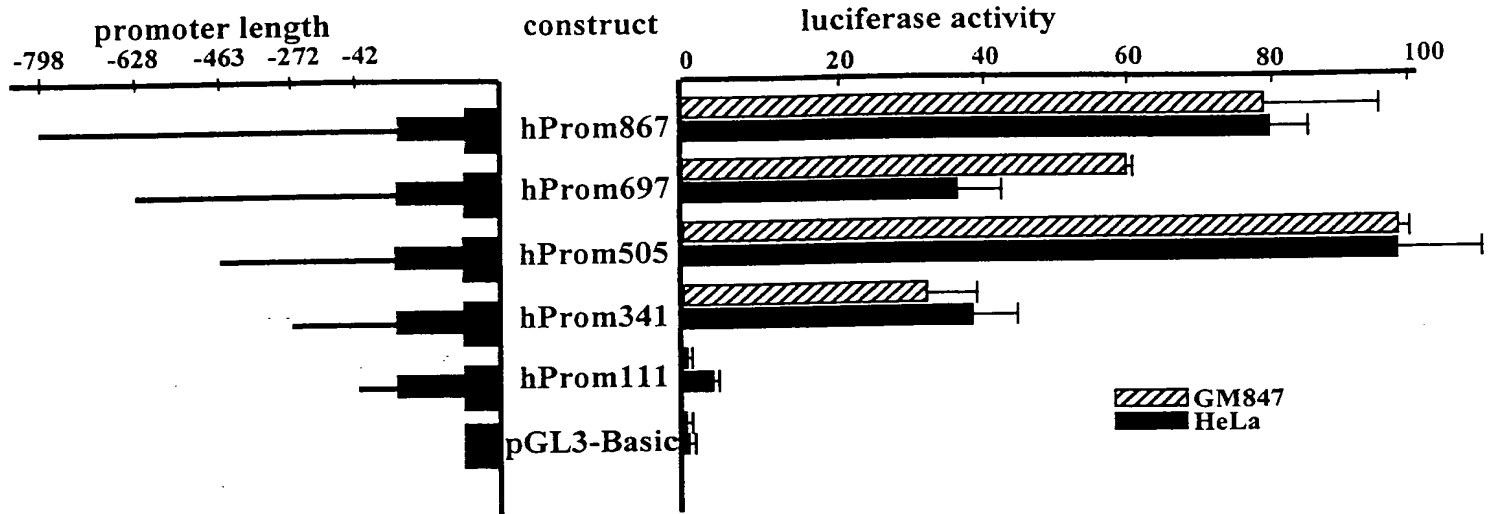
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^{PPAR/ELP} ^{GR}
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-431 tactgtgtggccatgcaactacaagagatccgcgcctgtcctctgtctctc
^{API/GCN4/Zeste} ^{mProm458} ^{SP-1}
-381 aaattctggaattaagaatttgcgcacatttcccccacttccacccccgg
^{C/EBPalpha, beta} ^{AP-2}
-331 ctgtggag^{mProm418}dggtgctgggttgaaggtggaatttttttttttttttt
^{p300}
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^{CP1}
-231 atattcagaacaacagcctcagagatgtgcgtgcgtgcgtgtgtgtgtg
^{mProm267}
-181 tgtagtgtgtgtgtgtctcacagacaagaacagatttattattattttt
^{GR} ^{F2F/Pt-1a}
-131 tattattatttttttgcgaagtgcctagagaaagtggtgggaagcggg
^{mProm208} ^{IRF-1,2} ^{GCN4/AP1} ^{AP-2} ^{c-Ets-2} ^{mProm136}
^{AP-2} ^{c-Ets-2} ^{CCAAT Box/AP-1}
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template
cgctgttttctctgcgtgacttccagcgg^{mProm}

Fig 4

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a) Human promoter activity in GM847 & HeLa cells



b) Mouse promoter activity in A9 & Swiss3T3 cells

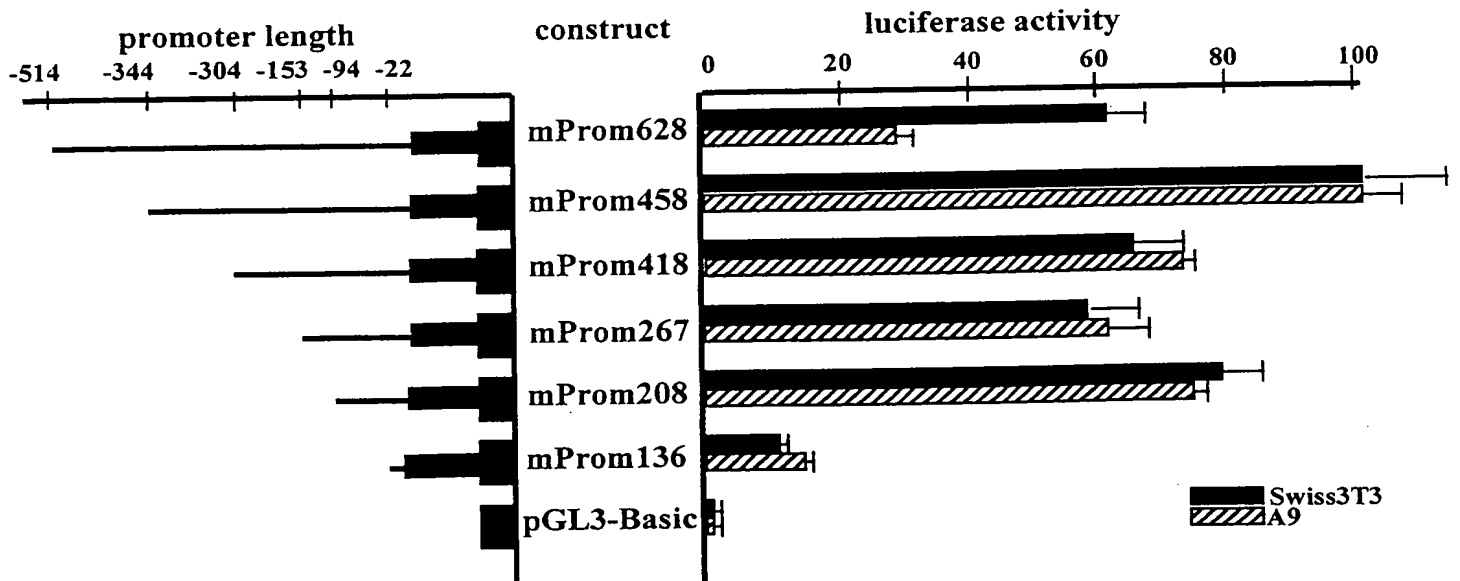


Fig. 5

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Oligo's Used: human

Name	Sequence	Comments
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hTR14	GGATCCTACGCCCTTCTCAGTTAGGGTTAG	hTR5 with BamHI site
hTR13F	ACTGAGCCGAGACAAGATTC	
hTR17F	GGATCCACTGAGCCGAGACAAGATTC	hTR13F with BamHI site
hTR10F	AGCTACTCAGGAGGCTGAGA	
hTR20F	GCGCTCGAGAGCTACTCAGGAGGCTGAGA	hTR10F with XhoI site plus gcg clamp
hTR11F	CATCAAGACACAGCACTACT	
hTR21F	GCGCTCGAGCATCAAGACACAGCACTACT	hTR11F with XhoI site plus gcg clamp
hTR6F	GTCTGGTCTGCAGAGGATAG	
hTR22F	GCGCTCGAGGTCTGGTCTGCAGAGGATAG	hTR6F with Xho site plus gcg clamp
hTR5	TACGCCCTTCTCAGTTAGGGTTAG	
hTR23R	CGCAAGCTTTACGCCCTTCTCAGTTAGGGTTAG	hTR5 with HindIII site plus cgc clamp
hTRe	CTGAGCTGTGGGACGTGCAC	
hTRf	AGACGGGAGAACCCACGCAG	
hTRg	CTCGGCTCACACATGCAGTT	
hTRh	TCTGCAGAGCAGGAACTAAGT	
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TRC3R	GGCGAACGGGCCAGCAGCTGACATT	

Oligo's Used: Mouse

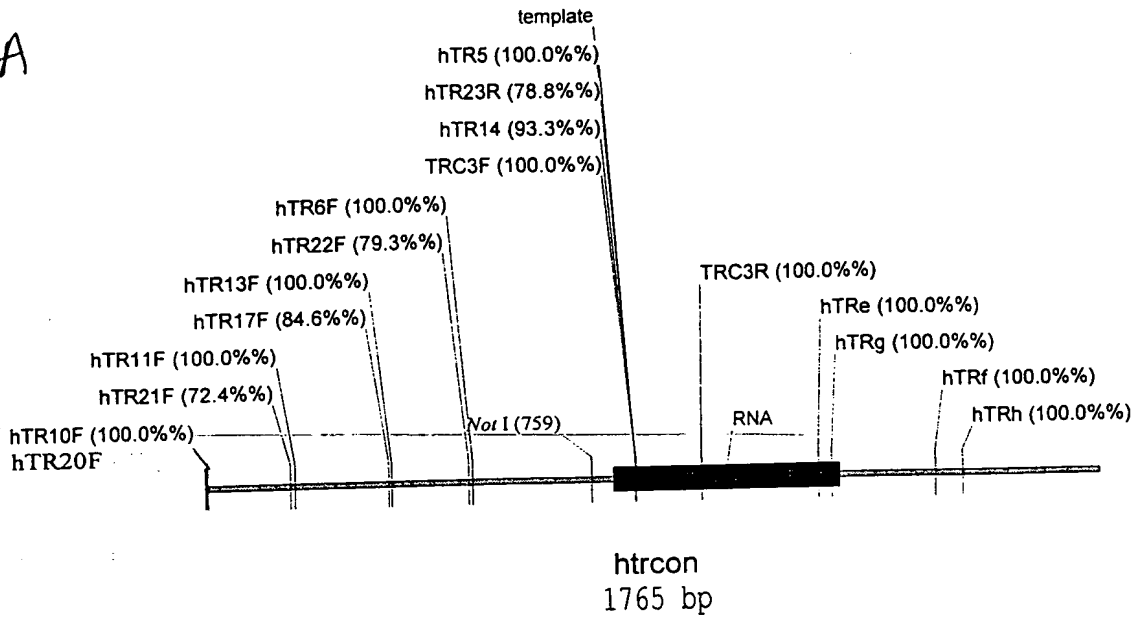
Name	Sequence	Comments
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mtr25f	GCGCTCGAGGTGTCTCACAGCAAGAAACA	This is mtr16f with XhoI site plus gcg clamp
mTR17F	GTGACTGGCTAGGAAGAGTG	
mtr26f	GCGCTCGAGGTGACTGGCTAGGAAGAGTG	This is mtr17f with XhoI site plus gcg clamp
mTR18F	TGTGACCTTGAACACAGAC	
mtr27f	GCGCTCGAGTGTGACCTTGAACACAGAC	This is mtr18f with XhoI site plus gcg clamp
mTR19F	GGAAGGTTGAAGGTGGAA	
mtr28f	GCGCTCGAGGGACTGGGTTGAAGGTGGAA	This is mtr19f with XhoI site plus gcg clamp
mTR20F	TGCGCCACTTTTCCCCACTT	
mtr29f	GCGCTCGAGTGCGCCACTTTTCCCCACTT	This is mtr20f with XhoI site plus gcg clamp
mTRr1	CCGCTGGAAGTCAGCGAGAA	
mtr30r	CGCAAGCTTCCGCTGGAAGTCAGCGAGAA	This is mTRr1 with HindIII site plus cgc clamp
mTR36F	GCGCTCGAGTCGACCAATCAGCGCGGCCAT	This is Xho I site PCR primer plus gcg clamp
mTRr1	CCGCTGGAAGTCAGCGAGAA	
mTRf1	TCGACCAATCAGCGCGGCCAT	

Fig. 6

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A



B

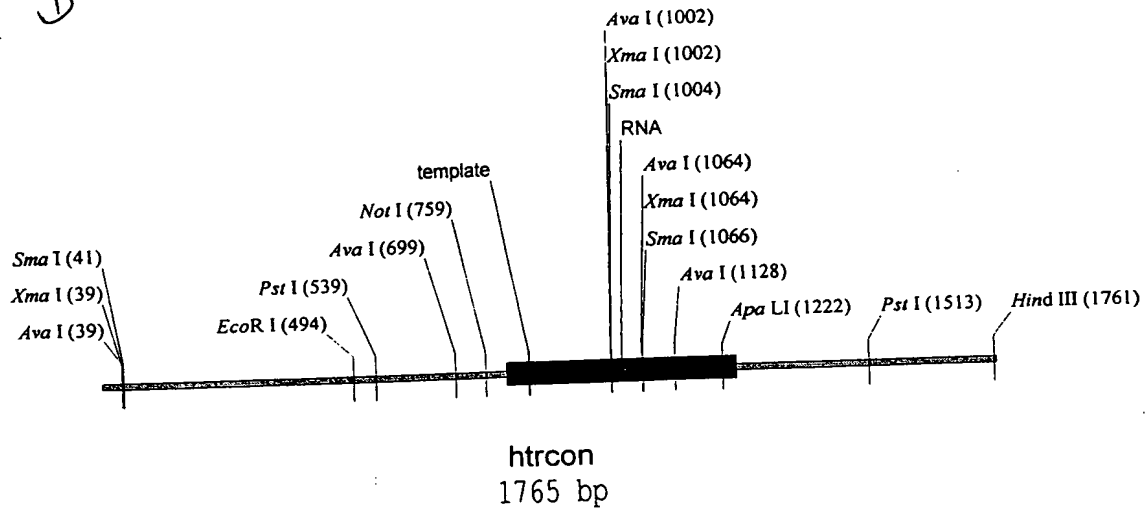
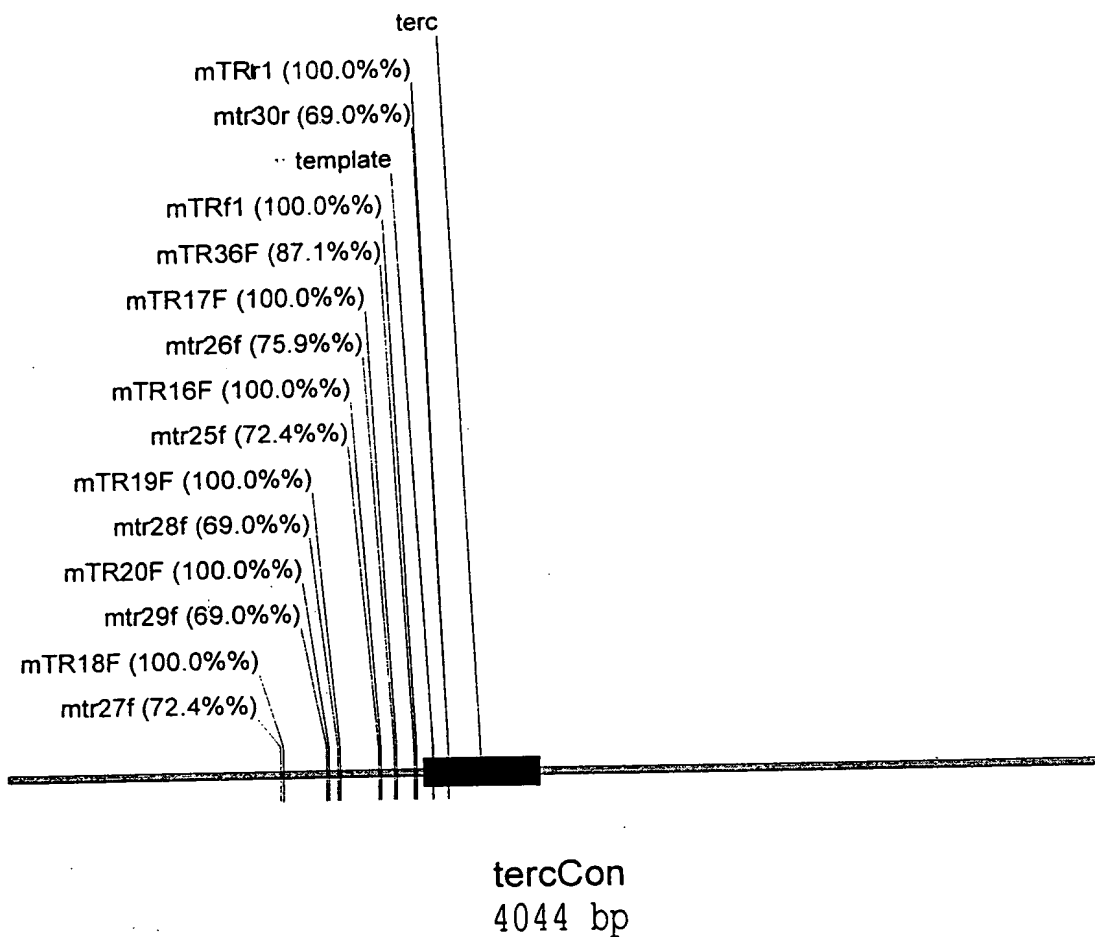


Fig. 7

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A



B

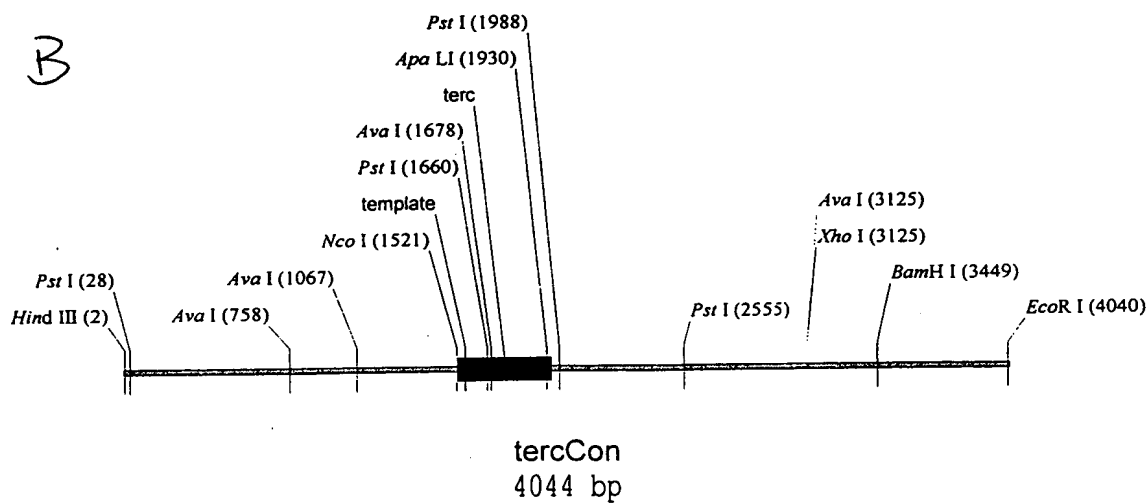


Fig. 8

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